masheff, S. N. (1989) *Biochemistry 28*, 5589-5599.
Melki, R., Carlier, M.-F., & Pantaloni, D. (1990) *Biochemistry 29*, 8921-8932.
O'Brien, E. T., Voter, W. A., & Erickson, H. P. (1987) *Biochemistry 26*, 4148-4156.
Olmsted, J. B., & Borisy, G. G. (1973) *Biochemistry 12*, 4282-4289.
Sherline, P., Leung, J. T., & Kipnis, D. M. (1975) *J. Biol. Chem. 250*, 5481-5486.
Sternlicht, H., & Ringel, I. (1979) *J. Biol. Chem. 254*, 10540-10550.
Stewart, R. J., Farrell, K. W., & Wilson, L. (1990) *Biochemistry 29*, 6489-6498.
Wilson, L. (1970) *Biochemistry 9*, 4999-5007.
Wilson, L., & Farrell, K. W. (1986) *Ann. N.Y. Acad. Sci. 466*, 690-708.
Wilson, L., Snyder, K. B., Thompson, W. C., & Margolis, R. L. (1982) *Methods Cell Biol. 24*, 159-169.
Wilson, L., Miller, H. P., Jordan, M. A., & Farrell, K. W. (1985) in *Microtubules & Microtubule Inhibitors* (De Brabander, M., & De May, J., Eds.) pp 71-78, Elsevier Science Publishers B. V., Amsterdam.

# $Ca^{2+}$ Dependence of the Interactions between Protein C, Thrombin, and the Elastase Fragment of Thrombomodulin. Analysis by Ultracentrifugation†

Philip H. Olsen,‡ Naomi L. Esmon,§ Charles T. Esmon,§,‖ and Thomas M. Laue*,‡

*Department of Biochemistry, University of New Hampshire, Durham, New Hampshire 03824, Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104, Departments of Pathology and of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190, and Howard Hughes Medical Institute, Oklahoma City, Oklahoma 73104*

*Received July 19, 1991; Revised Manuscript Received October 7, 1991*

ABSTRACT: The two-way and three-way interactions among active-site-blocked bovine thrombin, bovine protein C, and the elastase fragment of rabbit thrombomodulin (elTM) were examined by analytical ultracentrifugation at 23.3 °C in 100 mM NaCl, 50 mM Tris (pH 7.65), and 1 mM benzamidine, in the presence of 0 to 5 mM calcium chloride. Thrombin and elTM form a tight ($K_d < 10^{-8}$ M) 1:1 complex in the absence of $Ca^{2+}$ that weakens with the addition of $Ca^{2+}$ ($K_d \approx 4$ μM in 5 mM $Ca^{2+}$). Without $Ca^{2+}$, thrombin and protein C form a 1:1 complex ($K_d \approx 1$ μM) and what appears to be a 1:2 thrombin–protein C complex. The $K_d$ for the 1:1 complex weakens over 100-fold in 5 mM $CaCl_2$. Protein C and elTM form a $Ca^{2+}$-independent 1:1 complex ($K_d \approx 80$ μM). Nearly identical binding to thrombin and elTM is observed when active-site-blocked activated bovine protein C is substituted for protein C. Thrombin inhibited by diisopropyl fluorophosphate and thrombin inhibited by a tripeptide chloromethyl ketone exhibited identical behavior in binding experiments, suggesting that the accessibility of protein C to the substrate recognition cleft of these two forms of thrombin is nearly equal. Human protein C binds with lower affinity than bovine protein C. Ternary mixtures also were examined. Protein C, elTM, and thrombin form a 1:1:1 complex which dissociates with increasing [$Ca^{2+}$]. In the absence of $Ca^{2+}$, protein C binds to the elTM–thrombin complex with an apparent $K_d \approx 1$ μM. Nearly identical binding to elTM–thrombin was observed for activated protein C and protein C, suggesting that there is little discrimination between substrate and product. This was confirmed kinetically. Activated protein C is a potent inhibitor of its own formation both by thrombin alone ($K_i \approx 0.5$ μM) and by thrombin bound to elTM ($K_i \approx 4$ μM). Bovine protein C lacking the 41 amino acid γ-carboxyglutamic acid containing peptide (Gla domain) exhibited virtually no interaction with either elTM or elTM–thrombin, whereas the Gla domain competed effectively and specifically with protein C in these interactions. Finally, binding of the Gla domain to elTM–thrombin was observed. These results indicate that the Gla domain may be involved directly in the binding of protein C to both thrombin and elTM.

Thrombomodulin (TM),[1] an endothelial cell surface protein, is a cofactor for the activation of protein C by thrombin [for a review, see Esmon (1989)]. TM retains activity following solubilization with detergents, but the soluble form exists predominantly in the form of large aggregates (Winnard et al., 1989), making physical analyses difficult. However, limited proteolysis of TM by elastase yields a soluble fragment, termed elTM, which consists of the six epidermal growth factor-like domains of thrombomodulin (residues 234-486; Stearns et al.,

† This work has been supported in part by Grants BBS 86-15815 and DIR 90-02027 from the National Science Foundation (T.M.L.), Grants-in-Aid 85-0781 and 87-1092 from the American Heart Association (T.M.L.), and Grants R01 HL29807 and R37 HL30340 from the National Institutes of Health (C.T.E.). C.T.E. is an investigator of the Howard Hughes Medical Institute. P.H.O. is a recipient of a Predoctoral Fellowship Award from the American Heart Association, New Hampshire Affiliate. This is Scientific Contribution No. 1738 from the New Hampshire Agricultural Experiment Station.

* To whom correspondence should be addressed at the Department of Biochemistry, Spaulding Life Sciences Building, University of New Hampshire, Durham, NH 03824.
‡ University of New Hampshire.
§ Oklahoma Medical Research Foundation.
‖ University of Oklahoma Health Sciences Center and Howard Hughes Medical Institute.

[1] Abbreviations: EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Gla, γ-carboxyglutamic acid; PPACK, D-phenyl-L-prolyl-L-arginine chloromethyl ketone; DEGR, dansyl-L-glutamylglycyl-L-arginine chloromethyl ketone; TM, rabbit thrombomodulin; elTM, elastase fragment of rabbit thrombomodulin; elTM–thrombin, 1:1 complex of the elastase fragment of rabbit thrombomodulin and bovine thrombin; APC, activated bovine protein C; gdPC, protein C lacking the N-terminal 42 amino acids (Gla domain); $M_z$, z-average molecular weight; EGF, epidermal growth factor.

0006-2960/92/0431-746$03.00/0 

1989) and retains cofactor activity (Kurosawa et al., 1987).

The effect of $Ca^{2+}$ on protein C activation by elTM-T is complicated. The rate of activation is minimal at 0 mM $CaCl_2$, increases to a maximum at 0.3 mM $CaCl_2$, and then decreases 25-fold upon further addition of $CaCl_2$ (Kurosawa et al., 1987). Comparable results have been obtained with other soluble fragments of TM produced using recombinant methods (Hayashi et al., 1990). On the basis of these kinetic data, it has been postulated that the interaction of protein C with TM is mediated by a $Ca^{2+}$ bridge. Direct physical evidence supporting such a hypothesis is lacking.

In this paper, analytical ultracentrifugation is used to survey the effect of $Ca^{2+}$ on the interactions between elTM, thrombin, and either protein C (both bovine and human) or active-site-inhibited activated protein C (APC). The role of the protein C Gla domain in the interaction of protein C with thrombin and elTM is examined by direct binding experiments, by competition studies, and by examining the interactions of Gla-domainless bovine protein C (gdPC).

## Materials and Methods

*Solution Reagents.* Calcium chloride hydrate, 99.9+ %, was purchased from Aldrich Chemical Co. DEGR and PPACK were purchased from Calbiochem. The chromogenic substrate S-2238 was purchased from Helena Laboratories. All other buffer components were of reagent grade.

*Protein Preparation.* The following proteins and peptides were purified according to published methods: bovine thrombin (Owen et al., 1974), rabbit elTM (Kurosawa et al., 1987), bovine protein C (Walker et al., 1979), Gla-domainless bovine protein C (gdPC) and the Gla peptide (Esmon et al., 1983), and human protein C and bovine activated protein C (APC) (Vigano-D'Angelo et al., 1986). DEGR-inhibited bovine activated protein C ($APC_i$) and PPACK-inhibited thrombin used in kinetic assays were prepared by incubation with a large molar excess of inhibitor followed by removal of excess inhibitor by chromatography over a Mono Q (0.5 × 5 cm) column (Pharmacia LKB) for $APC_i$ or dialysis for the PPACK–thrombin.

Proteins were prepared for analytical ultracentrifugation by dialysis in buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.65, and 1 mM benzamidine) individually or as equimolar mixtures. Before dialysis, thrombin and protein C (and its derivatives) were treated with PPACK and DEGR, respectively, to minimize degradation during handling. Proteins were dialyzed (1000:1 v/v) as described previously (Laue et al., 1984) against the above buffer containing 2 mM EDTA, 0.3 mM $CaCl_2$, and 5 mM $CaCl_2$, or against Chelex 100 treated buffer that had Chelex 100 beads (Bio-Rad) confined in separate dialysis bags. Protein integrity was assessed using SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) as described previously (Laue et al., 1984).

*Protein Concentrations.* Protein molar concentrations were estimated using approximate molecular weights and the following extinction coefficients ($1\%^{-1}$ $cm^{-1}$): bovine thrombin, 21.0 (Owen et al., 1974); elTM, 6.0 (Kurosawa et al., 1987); bovine protein C, 13.7 (Kisiel et al., 1976); human protein C, 14.5 (Kisiel, 1979); gdPC, 13.7 (Esmon et al., 1983). The extinction coefficient for APC was assumed to be 13.7.

*Sedimentation Velocity.* Sedimentation velocity experiments were performed with both the Beckman Model E and the Beckman XLA analytical ultracentrifuges, at speeds of 44 000 and 60 000 rpm, respectively, using 4-hole rotors, 12-mm double-sector centerpieces, and at a constant temperature between 20.0 and 21.0 °C. Data were collected directly into a computer using absorbance optics ($\lambda$ = 280 nm). Proteins were dialyzed against buffer with 2 mM EDTA but containing no benzamidine, and the dialysate was used in the cell reference sectors. PPACK-inhibited thrombin, bovine protein C, and elTM were examined at concentrations of 0.1, 0.2, and 0.3 mg/mL, respectively. A mixture of thrombin and elTM was examined at final concentrations of each protein of 0.05 and 0.15 mg/mL, respectively.

Apparent sedimentation coefficients were determined and corrected to standard conditions as described (Chervenka, 1970) using the tabulated density and viscosity data (Weast, 1977). Sedimentation coefficient distribution functions were generated (Hayes & Laue, 1990; Hayes, 1991; unpublished results) and extrapolated according to the method of Van Holde and Weischet (1978). No correction was made for protein concentration since at these concentrations less than 1% error is likely (Teller, 1979). Anhydrous frictional coefficient ratios ($f/f_0$) for thrombin, elTM, protein C, and the elTM–thrombin complex were determined using $f$ calculated from the molecular weights and sedimentation coefficients and $f_0$ estimated according to the method of Teller (Teller, 1976; Teller et al., 1979). Frictional coefficient ratios for hydrated proteins ($f/f_H$), where $f_H$ is the frictional coefficient of a hydrated sphere, were calculated over a range of degrees of hydration (in grams of $H_2O$/gram of protein). Prolate ellipsoid axial ratios, $a/b$, were determined from a power series approximation of tabulated data (Cantor & Schimmel, 1980) using the equation:

$$a/b = 1.0 + 2.346X^{1/2} + 8.297X + 8.400X^2 - 0.4589X^3 + 0.0314X^4 \quad (1)$$

where $X = f/f_H - 1$. The coefficients in the power series were determined using nonlinear least-squares analysis (Johnson & Frasier, 1985).

*Sedimentation Equilibrium.* Centrifugation was performed at 23.3 °C and at the rotor speeds indicated in a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control, RTIC temperature controller, Rayleigh interference optics, and a HeNe laser light source (Williams, 1978). Meniscus depletion experiments (Yphantis, 1964) used externally loaded cells (Ansevin et al., 1970). Short-column (0.7 mm) experiments (Yphantis, 1960) used charcoal-filled Kel-F centerpieces. For earlier experiments, data were recorded on Kodak Technical Pan film and read on an automated plate reader (Laue & Yphantis, 1979; Laue, 1981). Later experiments used a television camera based, on-line data acquisition and analysis system (Laue, 1981; Laue, unpublished experiments). Data were collected at intervals after the estimated equilibrium time and tested for equilibrium by subtracting successive scans (Yphantis, 1964). Blank correction was performed as described previously (Yphantis, 1964; Laue et al., 1984).

For meniscus depletion experiments, free $Ca^{2+}$ concentrations were established by dialysis. For short-column experiments, buffer containing concentrated $CaCl_2$ was added directly to the solution just prior to centrifugation, leading to a small (<5%) uncertainty in the free $Ca^{2+}$ concentration. Gla peptide was dialyzed against the same buffer as the proteins and then added directly to the solution and reference sectors. Redistribution of $Ca^{2+}$ or Gla peptide was negligible at the concentrations and rotor speeds used. All additions were made so that a constant final protein concentration resulted.

*Data Analysis.* For each sample, between 75 (short column) and 400 (meniscus depletion) data points were acquired at a maximum spacing of 10 $\mu$m (cell coordinates). Blank correction and data editing were performed using REEDIT (kindly provided by D. Yphantis). Nonlinear least-squares analyses

were performed using NONLIN (Johnson et al., 1981). The 65% confidence interval determined by NONLIN is only an estimate of the precision of the fit and does not reflect the accuracy of a model (Johnson et al., 1981). Models found useful included that for nonideal self-association (Johnson et al., 1981; Laue et al., 1984; Luckow et al., 1989)

$$Y(r) = \delta + \alpha \exp\{\sigma\xi - 2\beta[Y(r) - \delta]\} + \sum_{N>1} \alpha^N K_N \exp\{N[\sigma\xi - 2\beta[Y(r) - \delta]]\} \quad (2)$$

as well as that for an ideal heteroassociation (Luckow et al., 1989)

$$Y(r) = \delta + \alpha_A \exp(\sigma_A\xi) + \alpha_B \exp(\sigma_B\xi) + \sum_{N\geq 1} \alpha_A\alpha_B^N K_{AB}^N \exp[(\sigma_A + N\sigma_B)\xi] \quad (3)$$

where $Y(r)$ is the fringe displacement (in fringes) at a radius $r$, $\delta$ is the base-line offset, $\alpha$ is the monomer activity at the arbitrary reference radius $r_0$, $\sigma$ is the reduced molecular weight, $\xi = (r^2 - r_0^2)/2$, $\beta$ is the nonideality coefficient, $N$ is the stoichiometry of the association, $K$ is the association constant of the complex, and the subscripts A and B refer to the reacting species in the heterocomplex (Johnson et al., 1981). The model for a single, ideal thermodynamic component ("single component") used eq 2 with $\beta = 0$ and $\ln K_N = -1 \times 10^4$. This same equation was used to model ideal ($\beta = 0$) heterodimer formation as a "pseudo self-association" when the difference in $\sigma$ between the interacting components was less than 2.5 $cm^{-2}$ (Laue et al., 1984; Luckow et al., 1989).

*Buffer Densities and Protein Partial Specific Volume.* Buffer densities, determined at 23.3 °C using a density meter (Mettler/Paar Model DMA 02 D) and published methods (Kratky et al., 1973), were 1.0040 g/mL for buffer containing either 2 mM EDTA or 0.3 mM $CaCl_2$ and 1.0044 g/mL for buffer containing 5 mM $CaCl_2$. Chelex-treated buffer was assumed to have a density of 1.0040 g/mL. The partial specific volumes calculated from amino acid and carbohydrate compositions (McMeekin & Marshall, 1952; Durschlag, 1986) were as follows: bovine protein C, human protein C, APC, and gdPC, 0.712 mL/g; elTM, 0.692 mL/g; bovine thrombin, 0.731 mL/g; and the Gla peptide of protein C, 0.702 mL/g (Olsen, 1991).

*Conversion of Units.* The conversion from the fringe displacement concentration scale to molar concentration scale was done as described previously (Laue et al., 1984; Luckow et al., 1989) using a specific fringe displacement of 3.52 fringes-L/g. Dissociation energies were calculated using $\Delta G°' = -RT \ln K_d$, where $R$ is the universal gas constant, $T$ is temperature, and $K_d$ is the reciprocal of the best-fit association constant returned by NONLIN (Johnson et al., 1981).

*Assay of the Inhibition of Protein C Activation by Activated Protein C.* Protein C activation rates were determined as described previously (Esmon et al., 1983). Assays were conducted under optimal conditions for activation as follows. Protein C activation by thrombin (5 nM) alone was done in 1.0 mM EDTA, 0.1 M NaCl, 0.02 M Tris-HCl, and 0.1% gelatin, pH 7.5, at 37 °C for 10 min. Protein C activation by thrombin and elTM (20 nM) was carried out similarly, but the reaction time was decreased to 5 min and 0.3 mM $CaCl_2$ was used instead of EDTA. To ensure that the thrombin was maximally bound by elTM, a mixture of PPACK-inhibited thrombin (15 nM, >99% inhibition) and thrombin (0.08 nM) was used. After incubation, both reactions were stopped by the addition of antithrombin III, and APC production was measured by observing the rate of hydrolysis of D-Phe-pipecoyl-Arg-*p*-nitroanilide (S-2238) that was at an initial concentration of 0.2 mM (Esmon et al., 1983). The reaction was monitored at 405 nm with a Molecular Devices $V_{max}$ spectrophotometer at room temperature (21–25 °C). Residual activity of DEGR-inhibited APC was subtracted from the final results.

Table I: Properties of the Isolated Components in 2.0 mM EDTA

| protein[a] | concn[b] (μM) | speed[c] (×10⁻³ rpm) | $M_r$[d] | $BM_r$[e] (mL/g) | rms[f] (fr) |
|---|---|---|---|---|---|
| BPC | 9.42 | 24, 28 | 62 400 (62 200–62 700) | 0 | 0.014 |
| gdBPC | 15.0 | 20, 24 | 54 600 (54 200–54 900) | 8 (6–10) | 0.019 |
| HPC | 16.6 | 20, 24 | 61 400 (60 900–61 900) | 0 | 0.030 |
| gdHPC | 10.0 | 20, 24, 28 | 54 100 (53 500–54 700) | 13 (8–18) | 0.024 |
| elTM | 18.3 | 20, 24, 28, 32 | 43 900 (34 700–35 100) | 0 | 0.022 |
| BTp | 42.0 | 20, 24, 28, 32 | 38 800 (38 600–39 000) | 0 | 0.020 |

[a] Abbreviations for protein names: BPC, bovine protein C; gdBPC, bovine Gla-domainless protein C; HPC, human protein C; gdHPC, human Gla-domainless protein C; elTM, elastase fragment of rabbit thrombomodulin; BTp, PPACK-inhibited bovine thrombin. [b] Maximum cell loading concentration. [c] Simultaneous fit to data acquired at the listed rotor speeds. [d] Molecular weight determined from $\sigma$ using partial specific volumes and densities listed under Materials and Methods. 65% confidence interval (in parentheses). [e] Product of the second virial coefficient and the fitted molecular weight. [f] Square root of the variance of fit in units of fringe displacement (fr).

## Results

*Sedimentation Equilibrium of the Individual Proteins.* The molecular weights of the individual proteins in buffer containing 2 mM EDTA are presented in Table I. No significant self-association ($K_d > 1.5$ mM) could be detected, even with the addition of up to 5 mM $CaCl_2$, thus simplifying the study of the heteroassociations (Olsen, 1991).

Bovine and human protein C consistently exhibit nearly identical molecular weights (Table I). This is somewhat unexpected since human protein C has a glycosylation variation leading to an expected difference of 5000 in molecular weight (Kisiel et al., 1976; Kisiel, 1979; Grinnell et al., 1991). Identical molecular weights were determined from several preparations of each protein, and it is not understood at this time why no difference was observed. Activation of protein C leads to no detectable change in molecular weight (Olsen, 1991). This suggests that the activation peptide remains noncovalently associated with the protein. However, an alternative explanation is that the loss in mass accompanying activation (≈1400 g/mol) is compensated by a slight (<0.1%) decrease in $\bar{v}$. The molecular weights determined for bovine and human gdPC are about 5% lower than expected (Esmon et al., 1983). The reason for this is unclear, but may result from a slight decrease in partial specific volume accompanying the removal of the Gla domain. Data for either gdPC were of sufficient quality to determine the second virial coefficient, which did not change detectably upon addition of $Ca^{2+}$ (Olsen, 1991).

The calculated amino acid chain molecular weight of elTM is 27 400 (Stearns et al., 1989). However, within the elTM sequence there are two potential Asn-linked glycosylation sites (residues 409 and 411). The molecular weight from sedimentation equilibrium (Table I) is consistent with a model of elTM in which both potential Asn-linked glycosylation sites are occupied with $M_r$ ≈3500 oligosaccharides (Kornfeld &

Table II: Interactions of PPACK-Inhibited Bovine Thrombin, Bovine Protein C, Bovine-Activated Protein C, and Gla-Domainless Bovine Protein C with elTM

| protein[a] | concn[b] ($\mu$M) | buffer addition[c] (mM) | speed[d] ($\times 10^{-3}$ rpm) | $M_r$[e] | $K_d$[f] ($\mu$M) | rms[g] (fr) |
|---|---|---|---|---|---|---|
| BTp + elTM | 21.5 | 2.0 EDTA | 20, 24 | 73 300 (72 500–74 000) | und[h] | 0.043[i] |
| | | 0.3 $Ca^{2+}$ | 20, 24 | 69 900 (68 700–71 000) | und[h] | 0.032[i] |
| | | | 20, 24 | 36 700[j] | 0.042 (0.01–0.18) | 0.032[k] |
| | | 5.0 $Ca^{2+}$ | 20, 24 | 36 700[j] | 4.19 (3.7–4.7) | 0.040[k] |
| BPC + elTM | 4.7 | 2.0 EDTA | 15, 20 | 48 100[j] | 82.0 (77.0–87.0) | 0.018 |
| | | 0.3 $Ca^{2+}$ | 15, 20 | 48 100[j] | 71.0 (66.0–75.0) | 0.019 |
| | | 5.0 $Ca^{2+}$ | 15, 20 | 48 100[j] | 69.0 (65.0–74.0) | 0.017 |
| BAPC[l] + elTM | 7.0 | none[m] | 20, 24, 28 | 48 100[j] | 35.0 (31.0–41.0) | 0.008 |
| | | 0.5 $Ca^{2+}$ | 20, 24, 28 | 48 100[j] | 53.0 (42.0–67.0) | 0.016 |
| | | 4.0 $Ca^{2+}$ | 20, 24, 28 | 48 100[j] | 52.0 (44.0–61.0) | 0.013 |
| gdBPC + elTM | 5.0 | 2.0 EDTA | 15, 20 | 49 400 (49 000–49 800) | nd[n] | 0.021 |
| | | 0.3 $Ca^{2+}$ | 15, 20 | 49 200 (48 800–49 600) | nd[n] | 0.020 |
| | | 5.0 $Ca^{2+}$ | 15, 20 | 51 800 (51 100–52 800) | nd[n] | 0.046 |

[a] Protein abbreviations given in Table I. [b] Cell loading concentration of each protein. [c] Proteins dialyzed against buffer containing the addition listed. [d] Simultaneous fit to data acquired at the listed rotor speeds. [e] Molecular weight and the 65% confidence interval (in parentheses). [f] Dissociation constant and the 65% confidence interval (in parentheses). [g] Square root of the variance of fit in units of fringe displacement. [h] und = no dissociation detected. [i] Modeled as a single ideal component (see Materials and Methods). [j] This value was held constant during the fit. [k] Modeled as pseudo-self-association (see Materials and Methods). [l] Short-column sedimentation results. See Materials and Methods for details. [m] Chelex-treated buffer and glassware (Olsen, 1991). [n] nd = no association detected.

Kornfeld, 1985). This would mean that elTM is 21% carbohydrate by weight which could explain the anomalously high apparent molecular weight observed for elTM by SDS–PAGE ($M_r \approx 50\,000$; Kurosawa et al., 1987).

The molecular weight of PPACK-inhibited thrombin measured by sedimentation equilibrium is within the experimental error of previous results (Baughman & Waugh, 1967; Owen et al., 1974) when the mass of the tripeptide inhibitor is taken into account.

*Interaction of elTM with Thrombin.* The interaction of elTM with thrombin in the *absence* of $CaCl_2$ is too strong to determine a reliable estimate of $K_d$ using the methods employed here (Table II). The molecular weight is exactly that expected for a 1:1 complex of elTM and thrombin. Under the conditions of these experiments, modeling suggests that the *upper* limit for $K_d$ is about $10^{-8}$ M (Laue et al., 1984). With the addition of 0.3 mM $CaCl_2$ to buffer, the data fit equally well to either of two models (see Materials and Methods, eq 1): (1) elTM–thrombin behaving as a single ideal component or (2) a monomer–dimer pseudo-self-association (Laue et al., 1984; Luckow et al., 1989). However, the molecular weight for the single component is somewhat lower than that anticipated for the 1:1 complex, suggesting that use of the interacting model is justified. The estimated $K_d$ is at the *lower* limit of detection using this experimental arrangement. The addition of more $Ca^{2+}$ further weakened the interaction between elTM and thrombin, such that at 5 mM $Ca^{2+}$ a significant fraction of the elTM–T complex is dissociated, even at the elevated protein concentrations employed here.

*Interaction of elTM and Protein C.* The interaction of elTM with either bovine protein C or APC is weak ($K_d \approx 70$ $\mu$M, using a pseudo-self-association model assuming a monomer $M_r$ = 48 100; Laue et al., 1984; Luckow et al., 1989) and relatively $Ca^{2+}$ insensitive (Table II). When human protein C is substituted for bovine protein C, binding to elTM is even weaker ($K_d \approx 250$ $\mu$M), but still $Ca^{2+}$-independent (Olsen, 1991). Due to its extreme weakness, no reliable $K_d$ could be determined for the interaction between elTM and gdPC (Table II). However, the observed molecular weight is somewhat higher than that expected for a noninteracting mixture of these proteins (44 800), suggesting that a very weak interaction ($K_d > 1$ mM) may be occurring between them. These data indicate that the interaction between protein C and elTM requires the Gla domain.

*Interaction of Thrombin and Protein C.* $CaCl_2$ was not required for the interaction between thrombin and protein C or APC (Figure 1). This was expected since thrombin activates protein C optimally in the absence of $Ca^{2+}$ ($K_m \approx 1.2$ $\mu$M). The addition of 3 mM $CaCl_2$ increases the $K_m$ to more than 60 $\mu$M (Esmon et al., 1983), and the decrease in molecular weight seen here is consistent with the kinetic observations. However, the data at 0 mM $Ca^{2+}$ (Figure 1) make it apparent that oligomers larger than 1:1 thrombin–protein C are formed, revealing a complexity not detected by kinetic analysis.

There is some uncertainty in the stoichiometry of the higher order complexes in the mixtures of protein C with thrombin. Numerous models were tried that might describe the higher oligomers (Olsen, 1991). On the basis of the criteria discussed in Luckow et al. (1989), the model that consistently fit the data best was

$$\text{thrombin} + C \rightleftarrows \text{thrombin}\cdot C + C \rightleftarrows \text{thrombin}\cdot C_2$$

This same model best described the interaction between thrombin and APC. Using this model, the [$Ca^{2+}$] dependence of the free energies for the dissociation in the first step of this scheme is presented in Figure 2, and that for the second step is presented in Figure 3. Comparison of these figures indicates

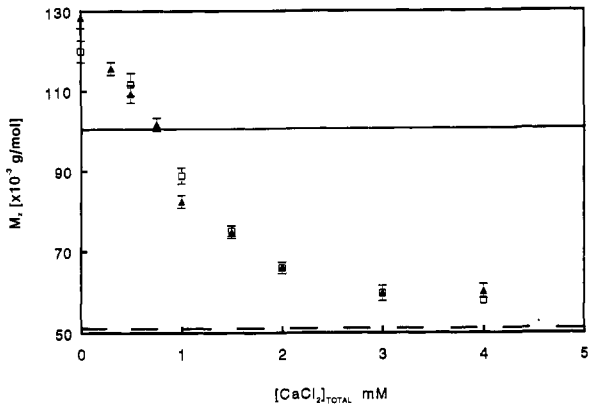


FIGURE 1: z-Average molecular weight of mixtures of protein C or activated protein C and thrombin as determined by short-column sedimentation equilibrium over a range of $CaCl_2$ concentrations. Samples were prepared for sedimentation as described under Materials and Methods. The thrombin and protein C (□) and the thrombin and activated protein C (▲) experiments were done at equimolar protein concentrations of 10 and 7.5 μM, respectively. The dashed line indicates the expected $M_z$ for no interaction between thrombin and protein C. The solid line represents the expected $M_z$ for a 1:1 association of thrombin and protein C. Each point represents a simultaneous fit of the data to three speeds: 20000, 24000, and 28000 rpm.

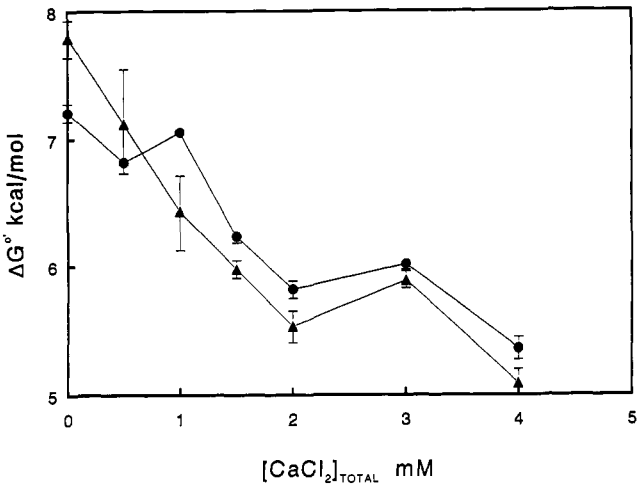


FIGURE 2: Free energy of dissociation of protein C from thrombin, i.e., thrombin–protein C ↔ thrombin + protein C (●), and of APC from thrombin, i.e., thrombin–APC ↔ thrombin + APC (▲). Values of the free energy were determined using eq 2 and the best-fit values from NONLIN for the dissociation constant as described under Materials and Methods.

that approximately the same amount of energy is required to dissociate the first and second protein C molecules from thrombin under low-$Ca^{2+}$ conditions. Nearly identical dissociation energies were determined for the first interaction when models describing other higher oligomers in the second interaction (e.g., $T_2$–protein $C_2$) were used for analysis, thus increasing our confidence in the values presented in Figure 2. Identical results are obtained when DFP-inhibited thrombin is used in place of PPACK-inhibited thrombin (Olsen, 1991). Substitution of human protein C for bovine protein C results in binding to thrombin with a similar [$Ca^{2+}$] dependence (Figure 1), and oligomers greater than a 1:1 thrombin–protein C complex are formed. However, human protein C exhibits binding affinity in 2 mM EDTA approximately an order of magnitude weaker than bovine protein C ($K_d \approx 12$ μM for human protein C versus 1.2 μM for bovine protein C).

It is evident from Figures 1–3 that APC binds to thrombin as well or even slightly better than protein C. This is somewhat

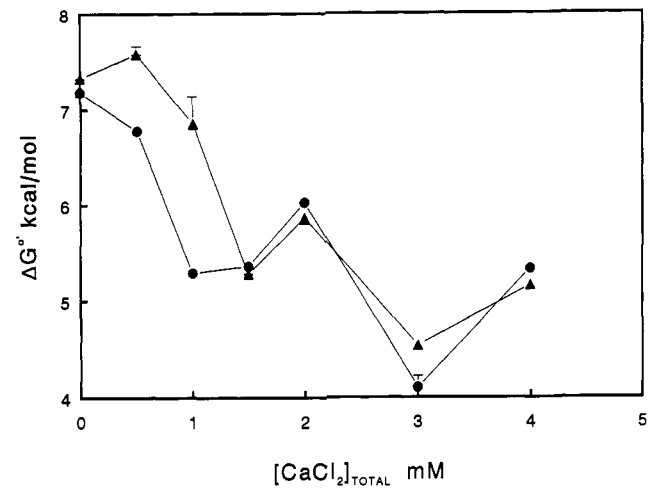


FIGURE 3: Free energy of dissociation of protein C from a thrombin–protein C complex, i.e., thrombin–protein $C_2$ ↔ thrombin–protein C + protein C (●), and of APC from thrombin–APC (▲). Values of the free energy were determined using eq 2 and the best-fit values from NONLIN for the dissociation constant as described under Materials and Methods.

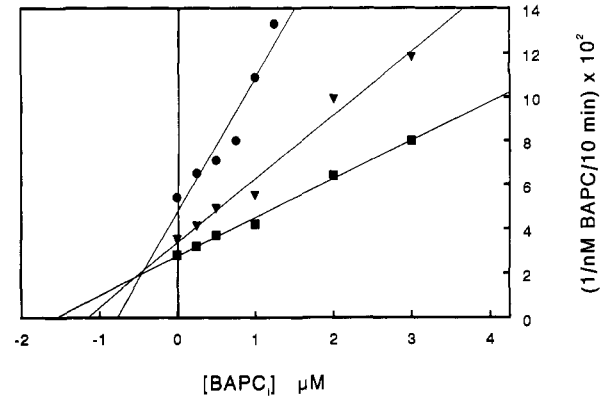


FIGURE 4: Activation of protein C by thrombin is inhibited by activated protein C. The reaction was carried out as described under Materials and Methods. The initial concentrations of protein C used were 0.4 μM (●), 0.8 μM (▼), and 1.2 μM (■). This is a representative Dixon plot of several trials. $BAPC_i$ = DEGR-inhibited bovine activated protein C.

surprising since APC is the product of the thrombin-catalyzed activation reaction and, thus, suggests that APC would be a potent inhibitor of protein C activation. Indeed, APC inhibits protein C activation with an apparent $K_i \approx 0.5$ μM (Figure 4), a value well below the apparent $K_m$ (1.2 μM; Esmon et al., 1983) for protein C under these conditions. The inhibition appears to be competitive, as would be expected if these molecules were binding to the same site. On the basis of the equilibrium data, under the conditions used here there would be insignificant levels of thrombin–protein $C_2$ or thrombin–$APC_2$ in the reaction mixtures.

*Interaction of elTM, Thrombin, and Protein C.* The interaction of protein C with thrombin and elTM was examined (Figure 5). It is clear that $Ca^{2+}$ is not required for complex formation and that the $Ca^{2+}$-dependent decrease in $M_z$ is similar to that for the interaction with thrombin alone (Figure 1). However, unlike thrombin alone, there is no indication of a second protein C binding site on elTM–T, since even in the absence of $Ca^{2+}$ there was no evidence for species larger than a 1:1:1 elTM–thrombin–protein C complex ($M_r \approx$ 136100). The simplest explanation for this observation is that protein C and elTM compete for one of the two sites on thrombin. In the absence of $Ca^{2+}$, elTM–T can be treated as

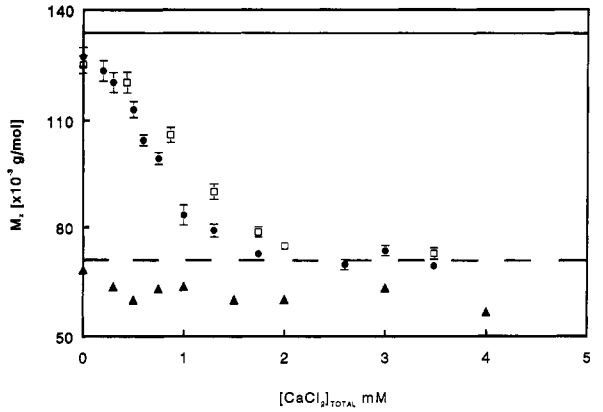


FIGURE 5: z-Average molecular weight, as determined by short-column sedimentation equilibrium, of equimolar mixtures of three proteins: 8 $\mu$M each of thrombin, elTM, and protein C (□); 7 $\mu$M each of thrombin, activated protein C, and elTM (●); and 7 $\mu$M for thrombin, Gla-domainless protein C, and elTM (▲). Samples were prepared as described under Materials and Methods. The dashed line represents the expected $M_z$ for no interaction between thrombin, protein C, and elTM. The solid line represents the expected $M_z$ for 1:1:1 association of thrombin, protein C, and elTM. Each point represents a simultaneous fit of the data to three speeds: 20 000, 24 000, and 28 000 rpm.

Table III: Estimates of the $K_d$ between elTM–Thrombin and Bovine Protein C, Bovine APC, or Bovine Gla-Domainless Protein C in the Absence of $Ca^{2+}$

| protein | concn[a] ($\mu$M) | speed[b] ($\times10^{-3}$ rpm) | $M_r$[c] | $K_d$[d] ($\mu$M) | rms[e] (fr) |
|---|---|---|---|---|---|
| BPC + elTM–BTp[f] | 8.0 | 15, 20, 24 | 67 600 | 1.70 (1.30–2.30) | 0.030 |
| BAPC + elTM–BTp | 7.0 | 15, 20, 24 | 67 600 | 0.890 (0.650–1.30) | 0.024 |
| gdBPC + elTM–BTp | 7.0 | 15, 20, 24 | 64 400 | 181 (166–201) | 0.012 |

[a] Cell loading concentration of each protein. [b] Simultaneous fit to data acquired at the listed rotor speeds. [c] This molecular weight was held constant during the fit. [d] Dissociation constant determined and the 65% confidence interval (in parentheses). [e] Square root of the variance of fit in units of fringes. [f] Protein abbreviations given in Table I; elTM–BTp, 1:1 complex of PPACK-inhibited bovine thrombin and the elastase fragment of rabbit thrombomodulin.

a single component (Laue et al., 1984; Luckow et al., 1989) whose interaction with protein C is fit best as a pseudo-self-association to dimer (Table III). In 0 mM $Ca^{2+}$, APC binds to elTM–T with equal or slightly greater affinity than protein C does (Figure 5, Table III). At 300 $\mu$M $Ca^{2+}$, the interaction was too complicated to fit to any simple model to determine $K_d$. Substitution of human protein C for bovine protein C results in a similar $Ca^{2+}$ dependence (Figure 5), but with a somewhat lower binding affinity (Olsen, 1991).

Though not large, there was an apparent and reproducible difference in the z-average molecular weight of elTM, thrombin, and protein C as compared to that of elTM, thrombin, and APC, particularly at [$CaCl_2$] above 0.5 mM (Figure 5). This suggests that elTM may somehow enhance the discrimination between protein C and APC. The use of DFP-inhibited thrombin in place of PPACK-inhibited thrombin yielded results that are indistinguishable from those in Figure 5 (Olsen, 1991), again suggesting that the lack of discrimination is not the result of the peptide inhibitor.

The Gla domain is required for the interaction of protein C with elTM–T. Not only is the [$Ca^{2+}$] dependence of the interaction eliminated by its removal (Figure 5) but also in 0 mM $Ca^{2+}$ the apparent $K_d$ of gdPC for the elTM–thrombin complex is more than 100-fold larger than that of protein C (Table III).

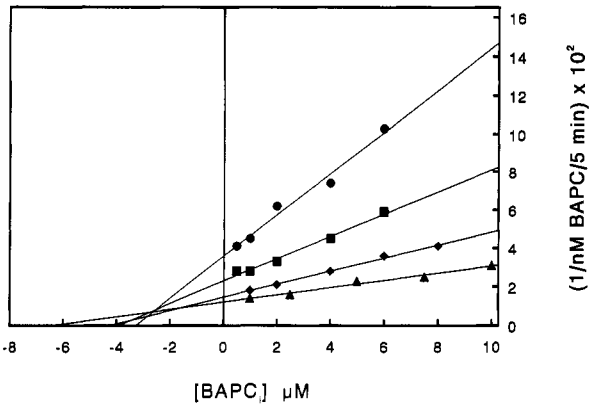


FIGURE 6: Activation of protein C by thrombin and elTM is inhibited by activated protein C. The activation was carried out as described under Materials and Methods. The initial concentrations of protein C used were 0.1 (●), 0.2 (■), 0.4 (♦), and 0.8 $\mu$M (▲). This is a representative Dixon plot of several trials. $BAPC_i$ = DEGR-inhibited bovine APC. There are no data points for the zero concentration of inhibitor condition since it was found that the protein C was sticking to the test tube walls due to the presence of $Ca^{2+}$.

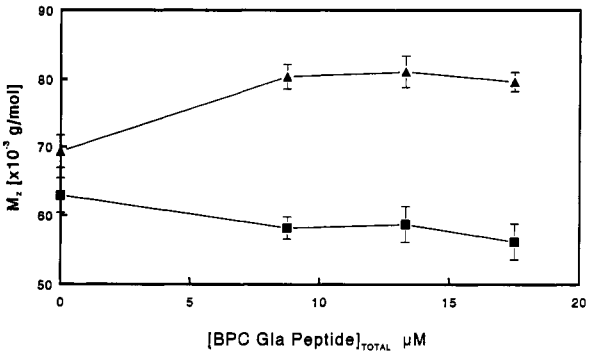


FIGURE 7: Effect of the Gla peptide on the z-average molecular weight of mixtures of elTM and thrombin (▲) and of elTM and activated protein C (■). Short-column sedimentation equilibrium was used to determine the $M_z$ at the indicated concentrations of the Gla peptide. Samples were prepared as described with Materials and Methods. The equimolar protein concentrations used were the following: for the elTM–thrombin mixture, 5 $\mu$M; for the elTM–activated protein C mixture, 5 $\mu$M. Each point represents the simultaneous fit of data acquired at 20 000, 24 000, and 28 000 rpm.

Under conditions of optimal protein C activation by elTM–T, APC is not as effective an inhibitor ($K_i \approx 3.0$–$5.0$ $\mu$M, Figure 6) as it is of protein C activation by thrombin alone (Figure 4). The inhibition appears to be competitive. As shown in Figure 5 at 300 $\mu$M $Ca^{2+}$, the decreased efficiency of APC as an inhibitor probably reflects the difference in the affinities of protein C and APC for elTM–thrombin.

*Effect of the Gla Peptide on the Interaction of elTM with Thrombin and Protein C.* The bovine Gla peptide, a 41 amino acid peptide, was generated by the partial chymotryptic digestion of bovine protein C (Esmon et al., 1983). Sedimentation equilibrium of the Gla peptide in the absence of $Ca^{2+}$ indicated that it exists in a monomer–dimer equilibrium with a dissociation constant of 4.5 $\mu$M (Olsen, 1991). The dimer has a molecular weight of about 9700.

Short-column sedimentation equilibrium experiments were conducted with mixtures of elTM and thrombin or elTM and APC, with the addition of several concentrations of the Gla peptide (Figure 7). Conditions were chosen so that the Gla peptide did not contribute significantly to the concentration gradient. Two Gla domains bind to elTM–thrombin as evidenced by the gain in molecular weight upon addition of the Gla peptide (Figure 7). On the other hand, the $M_z$ of the

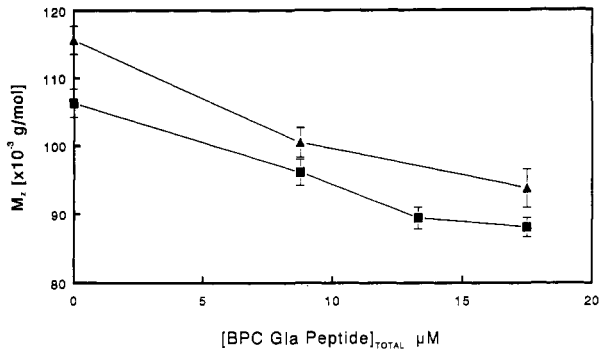


FIGURE 8: Effect of the Gla peptide on the z-average molecular weight of mixtures of thrombin and protein C (▲) and of thrombin and activated protein C (■). Short-column sedimentation equilibrium was used to determine the $M_z$ at the indicated concentrations of Gla peptide. Samples were prepared as described under Materials and Methods. The equimolar protein concentrations used were as follows: for the thrombin–protein C mixture, 5 μM; for the thrombin–activated protein C mixture, 5 μM. Each point represents the simultaneous fit of data acquired at 20 000, 24 000, and 28 000 rpm.

Table IV: Sedimentation Coefficients and Frictional Coefficient Ratios Determined in 2 mM EDTA for Thrombin, elTM, Bovine Protein C, and the Thrombin–elTM Complex

| protein[a] | $s_{20,w}$ (S) | $f/f_0$ |
|---|---|---|
| BTp | $3.3_9$ | $1.1_5$ |
| elTM | $2.5_3$ | $1.6_5$ |
| BPC | $4.0_2$ | $1.4_3$ |
| elTM–BTp | $4.2_0$ | $1.5_0$ |

[a] Protein abbreviations given in Tables I and III.

elTM and APC mixture decreased slightly with the addition of the Gla peptide, consistent with competition of the Gla peptide with APC for binding to elTM.

*Effect of the Gla Peptide on the Interaction of Thrombin and Protein C.* Titration of a mixture of thrombin and protein C with Gla peptide results in a decrease in $M_z$ (Figure 8). This decrease is consistent with competition between the Gla peptide and protein C or APC for thrombin binding. The specificity of this competition was tested by substituting the Gla domain from factor X for that from protein C. In the presence of an 8-fold molar excess (42 μM) of factor X–Gla domain, the z-average molecular weight decreased slightly from 115 000 in the absence Gla peptide to 105 000 (data not shown). Thus, although the Gla domains of the blood coagulation proteins are very similar (Furie & Furie, 1990), it appears that there may be Gla domain specificity in these interactions.

*Hydrodynamic Analysis.* Sedimentation coefficients and frictional coefficient ratios for thrombin, elTM, protein C, and the thrombin and elTM mixture are presented in Table IV. Indistinguishable results were obtained with the Model E and the XLA analytical ultracentrifuges. Expected prolate ellipsoid axial ratios for these molecules at various degrees of hydration are presented in Figure 9.

Bovine thrombin sediments as a symmetrical molecule. The value reported here is somewhat lower than that reported previously (3.7 S; Seegers et al., 1968) for uninhibited thrombin, with the difference possibly due to the prevention of active-site-mediated oligomerization in our sample. Bovine protein C is somewhat more asymmetric, suggesting that the EGF domains may form an asymmetric unit attached to the catalytic domain. The degree of asymmetry of protein C is in accord with other EGF-containing clotting proteins. The 1:3 molar ratio mixture of elTM and thrombin exhibited two boundaries that were not well resolved except by using the

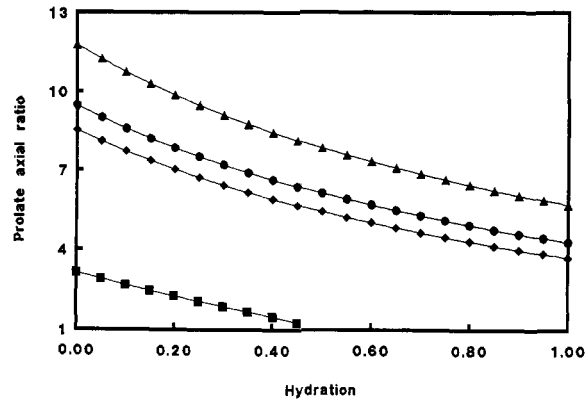


FIGURE 9: Prolate ellipsoid axial ratios of PPACK-inhibited bovine thrombin, elTM, bovine protein C, and the elmM–thrombin complex. Values of the prolate axial ratio were determined at the indicated degrees of hydration by using the procedure described under Materials and Methods. The axial ratios were computed by using the following data for molecular weights and sedimentation coefficients: for thrombin (■), 38 800 and $3.3_9$ S; for elTM (▲), 34 900 and $2.5_3$ S; for protein C (♦), 62 400 and $4.0_2$ S; for the thrombin and elTM complex (●), 73 700 and $4.2_0$ S.

method of Van Holde and Weischet (1978). Using their method, the faster boundary, presumably elTM–T, was 4.2 s, and the slower was about 2.5–2.6 s, corresponding to the excess elTM.

## Discussion

*Role of $Ca^{2+}$ in Protein C Activation.* The present results demonstrate that $Ca^{2+}$ is not required for any of the interactions in the protein C activation complex and argue against a model in which the enhanced activation of protein C by thrombin and elTM in 0.3 mM $CaCl_2$ results from the formation of a $Ca^{2+}$ bridge between protein C and elTM (Kurosawa et al., 1987). In fact, the addition of increasing quantities of $Ca^{2+}$ weakens the interactions between protein C and thrombin (Figures 1–3 and Figures 5–7) or between elTM and thrombin (Table II). Furthermore, the interaction of protein C (or APC) with elTM is weak and unaltered by the addition of $Ca^{2+}$ (Table II). Thus, while these observations do not rule out the presence of $Ca^2$ bridges, they do diminish their potential contribution to protein–protein binding. On the other hand, protein C is known to undergo conformational changes in the presence of $Ca^{2+}$ (Johnson et al., 1983). Thus, the more important role of $Ca^{2+}$ may be to bring about the conformational changes necessary for optimal activation. These changes are likely to be at the cleavage site and not detected accurately with the inactive thrombin derivatives used in this study.

*Role of the Gla Domain in Protein–Protein Interactions.* It is known that the Gla domains of several blood coagulation proteins are required for protein–phospholipid interactions (Nelsestuen & Broderius, 1977). Recently, evidence has been presented suggesting Gla mediation of protein–protein interactions in VIIa binding to tissue factor (Sakai et al., 1990). Likewise, it has been shown that the Gla domain of protein C is necessary for the optimal activation of protein C by endothelial cell surface thrombomodulin and thrombin (Esmon et al., 1983). The present data demonstrate that the Gla peptide from protein C is required for protein C interaction with either elTM (Table II) or elTM–T (Figure 5, Table III). Moreover, Gla peptide binding to elTM–T was demonstrated directly (Figure 7), as was its ability to compete specifically with protein C or APC for thrombin binding (Figure 8), even in the absence of $Ca^{2+}$. Taken together, these results suggest

that the Gla domain may contribute directly to the interactions of protein C with elTM and with thrombin. In the case of thrombin, it is possible that the Gla domain may be interacting with either or both of the two basic amino acid regions near thrombin's active-site cleft (Bode et al., 1990). However, it is unlikely that the Gla peptide is solely responsible for the interaction of protein C with thrombin, since excess peptide failed to completely eliminate the thrombin–protein C interaction (Figure 8).

*APC Inhibition of Protein C Activation.* It was surprising to find that thrombin could not discriminate between its substrate, protein C, and its product, activated protein C (Figure 2). Initially we postulated that PPACK might block substrate access to subsites in the substrate binding cleft of thrombin and that these sites might be important in distinguishing between protein C and APC. This hypothesis was tested with thrombin that had been inhibited with DFP. However, in all cases, DFP-inhibited thrombin yielded results indistinguishable from PPACK-inhibited thrombin (Olsen, 1991), suggesting either that merely disrupting thrombin in the vicinity of the catalytic site was sufficient to lose discrimination or that thrombin does not discriminate between binding C and APC. The kinetic results (Figures 4 and 6) support the latter conclusion by demonstrating that APC is a potent inhibitor of protein C activation and suggesting that APC can bind to thrombin more tightly than protein C. The physiological significance of this observation is not clear, but, in vivo, both protein C and activated protein C are at concentrations well below $K_m$ and, thus, it is unlikely that APC inhibits activation under physiological conditions.

*Hydrodynamics.* X-ray diffraction had shown that thrombin is nearly spherical and has a diameter of about 4.5 nm (Bode et al., 1989). If the estimate of the axial ratio from sedimentation of about 1.0 for thrombin is reasonable, then Figure 1 indicates that thrombin is hydrated at about 0.4–0.5 of $H_2O$/g of protein, which is slightly higher than most proteins (0.2–0.35 g of $H_2O$/g of protein; Kuntz & Kaufman, 1974). However, this hydration value is close to estimates for the hydration of chymotrypsin-like molecules (Harding, 1989).

The primary thrombin binding site on thrombomodulin has been localized to the fifth and sixth EGF-like domains of elTM (Kurosawa et al., 1988; Suzuki et al., 1989). Thus, thrombin is binding near the carboxyl terminus of elTM. Since the axial ratios indicate that elTM–T is less asymmetric than elTM, these data suggest that thrombin binds along the side of the more rod-like elTM. Thus, even though the interaction energies are nearly identical for protein C with elTM–thrombin and for protein C with thrombin alone, it is quite possible that the binding of protein C to elTM alone (Table II) contributes to the stabilization of the ternary complex.

The activation of protein C by elTM–thrombin can be thought of as a ligand-mediated, receptor-mediated interaction of an enzyme with its substrate and its product. The results of the current paper reveal the complexity of the interactions underlying the activation kinetics and demonstrate the utility of analytical ultracentrifugation for examining such systems.

## ACKNOWLEDGMENTS

We thank Theresa Ridgeway and Daryl Lyons for their technical expertise in the running of the Model E analytical ultracentrifuges and interferogram plate reading, David Hayes for help with the hydrodynamic analysis, David Yphantis for useful discussions and for provision of REEDIT and NONLIN, M. Martha Thomson, Thomas Moody, Andrew Laudano, and Donald Green for careful reading of the manuscript, and Clendon Brown and Jeff Box for protein purification.

**Registry No.** $Ca^{2+}$, 7440-70-2; thrombin, 9002-04-4; protein C, 60202-16-6; activated protein C, 42617-41-4.

## REFERENCES

Ansevin, A. T., Roark, D. E., & Yphantis, D. A. (1970) *Anal. Biochem. 34*, 237–261.

Baughman, D. J., & Waugh, D. F. (1967) *J. Biol. Chem. 242*, 5252–5259.

Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., & Hofsteenge, J. (1989) *EMBO J. 8*, 3467–3475.

Cantor, C. R., & Schimmel, P. R. (1980) in *Biophysical Chemistry*, Part II, p 561, W. H. Freeman, San Fransisco, CA.

Chervenka, C. H. (1970) *A Manual of Methods for the Analytical Ultracentrifuge*, pp 23–33, Spinco Division of Beckman Instruments, Inc., Palo Alto, CA.

Dittman, W. A., & Majerus, P. W. (1990) *Blood 75*, 329–336.

Durschlag, H. (1986) in *Thermodynamic Data for Biochemistry and Biotechnology* (Hinz, H.-J., Chapter 3, Ed.) pp 45–128, Springer-Verlag, New York.

Esmon, C. T. (1989) *J. Biol. Chem. 264*, 4743–4746.

Esmon, N. L., DeBault, L. E., & Esmon, C. T. (1983) *J. Biol. Chem. 258*, 5548–5553.

Furie, B., & Furie, B. C. (1990) *Blood 75*, 1753–1762.

Grinnell, B. W., Walls, J. D., & Gerlitz, B. (1991) *J. Biol. Chem. 266*, 9778–9785.

Harding, S. E., & Rowe, A. J., Eds. (1989) in *Dynamic Properties of Biomolecular Assemblies*, Chapter 2, Royal Society of London, London.

Hayashi, T., Zushi, M., Yamamoto, S., & Suzuki, K. (1990) *J. Biol. Chem. 265*, 20156–20159.

Hayes, D. (1991) Masters Thesis, University of New Hampshire, Durham, NH.

Hayes, D. B., & Laue, T. M. (1990) *Biophys. J. 59*, 109a.

Jackson, C. M., & Nemerson, Y. (1980) *Annu. Rev. Biochem. 49*, 765–811.

Johnson, A. E., Esmon, N. L., Laue, T. M., & Esmon, C. T. (1983) *J. Biol. Chem. 258*, 5554–5560.

Johnson, M. L., & Frasier, S. G. (1985) *Methods Enzymol. 117*, 301–342.

Johnson, M. L., Correia, J. J., Yphantis, D. A., & Halvorson, H. R. (1981) *Biophys. J. 36*, 575–588.

Kettner, C., & Shaw, E. (1981) *Methods Enzymol. 80*, 826–842.

Kisiel, W. (1979) *J. Clin. Invest. 64*, 761–769.

Kisiel, W., Ericsson, L. H., & Davie, E. W. (1976) *Biochemistry 15*, 4893–4900.

Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–664.

Kratky, O., Leopold, H., & Stabinger, H. (1973) *Methods Enzymol. 27*, 98–110.

Kurosawa, S., Galvin, J. B., Esmon, N. L., & Esmon, C. T. (1987) *J. Biol. Chem. 262*, 2206–2212.

Kurosawa, S., Stearns, D. J., Jackson, K. W., & Esmon, C. T. (1988) *J. Biol. Chem. 263*, 5993–5996.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Laue, T. M. (1981) Ph.D. Dissertation, University of Connecticut, Storrs, CT.

Laue, T. M., & Yphantis, D. A. (1979) *Biophys. J. 25*, 164a.

Laue, T. M., Johnson, A. E., Esmon, C. T., & Yphantis, D. A. (1984) *Biochemistry 23*, 1339–1348.

Luckow, E. A., Lyons, D. A., Ridgeway, T. M., Esmon, C. T., & Laue, T. M. (1989) *Biochemistry 28*, 2348–2354.

McMeekin, T. L., & Marshall, K. (1952) *Science 116*, 142–143.

Nelsestuen, G. L., & Broderius, M. (1977) *Biochemistry 16*, 4172-4176.
Olsen, P. H. (1991) Ph.D. Dissertation, University of New Hampshire, Durham, NH.
Owen, W. G., Esmon, C. T., & Jackson, C. M. (1974) *J. Biol. Chem. 249*, 594-605.
Sakai, T., Lund-Hansen, T., Thim, L., & Kisiel, W. (1990) *J. Biol. Chem. 265*, 1890-1894.
Seegers, W. H., McCoy, L., Kipfer, R. K., & Murano, G. (1968) *Arch. Biochem. Biophys. 128*, 194-201.
Stearns, D. J., Kurosawa, S., Sims, P. J., Esmon, N. L., & Esmon, C. T. (1988) *J. Biol. Chem. 263*, 826-832.
Stearns, D. J., Kurosawa, & Esmon, C. T. (1989) *J. Biol. Chem. 264*, 3352-3356.
Suzuki, K., Hayashi, T., Nishioka, J., Kosaka, Y., Zushi, M., Honda, G., & Yamamoto, S. (1989) *J. Biol. Chem. 264*, 4872-4876.
Teller, D. C. (1976) *Nature 260*, 729-731.
Teller, D. C., Swanson, E., & De Haen, C. (1979) *Methods Enzymol. 61*, 103-124.
Van Holde, K. E., & Weischet, W. O. (1978) *Biopolymers 17*, 1387-1403.
Vigano-D'Angelo, S., Comp, P. C., Esmon, C. T., & D'Angelo, A. (1986) *J. Clin. Invest. 77*, 416-425.
Walker, F. J., Sexton, P. W., & Esmon, C. T. (1979) *Biochim. Biophys. Acta 571*, 333-342.
Weast, R. C., Ed. (1977) *Handbook of Chemistry and Physics*, 58th ed., Chemical Rubber Publishing Co., Cleveland, OH.
Williams, R. C., Jr. (1978) *Methods Enzymol. 48*, 185-191.
Winnard, P. W., Esmon, C. T., & Laue, T. M. (1989) *Arch. Biochem. Biophys. 269*, 339-344.
Yphantis, D. A. (1960) *Ann. N.Y. Acad. Sci. 88*, 586-601.
Yphantis, D. A. (1964) *Biochemistry 3*, 297-317.
Yphantis, D. A., & Waugh, D. F. (1956) *J. Phys. Chem. 60*, 623-629.

# Molecular Modeling of the Domain Structure of C9 of Human Complement by Neutron and X-ray Solution Scattering

Kathryn F. Smith,[‡] Richard A. Harrison,[§] and Stephen J. Perkins*[,‡]

*Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K., and MRC Molecular Immunopathology Unit, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K.*

*Received August 1, 1991; Revised Manuscript Received October 11, 1991*

ABSTRACT: C9 is the most abundant component of the membrane attack complex of the complement system of immune defense. This is a typical mosaic protein with thrombospondin (TSR) and low density lipoprotein receptor (LDLr) domains at its N-terminus and an epidermal growth factor-like (EGF) domain at its C-terminus. Between these lies a perforin-like sequence. In order to define the arrangement in solution of these four moieties in C9, high-flux neutron and synchrotron X-ray solution scattering studies were carried out. The neutron radius of gyration $R_G$ at infinite contrast is 3.33 nm, and its cross-sectional $R_G$ ($R_{XS}$) is 1.66 nm. Similar values were obtained by synchrotron X-ray scattering after allowance for radiation effects. Stuhrmann analyses showed that the neutron radial inhomogeneity of scattering density $\alpha$ is $35 \times 10^{-5}$ from the $R_G$ data and $16 \times 10^{-5}$ from the $R_{XS}$ data. These values are typical for soluble glycoproteins and show no evidence for the existence of any large hydrophobic surface patches on free C9 that might form contacts with lipids. Indirect transformation of the neutron and X-ray scattering curves into real space showed that C9 had a maximum dimension estimated as $12 \pm 2$ nm, and this suggests that the lengths of 7-8 nm deduced from previous electron microscopy studies in vacuo are underestimated. Molecular modeling of the C9 scattering curves utilized small spheres in the Debye equation, in which the analyses were constrained by the known volumes of the four moieties of C9 and the known sizes of the TSR and EGF-like domains. The most likely models for C9 suggest that these four regions of C9 are arranged in a V-shaped structure, with an angle of 10° between the two arms, each of length 11.1 nm. This structure has a more hydrophobic character between the two arms. The scattering model is fully consistent with hydrodynamic sedimentation data on C9. Similar V-shaped hydrodynamic models could be developed for C6, C7, C8, and C9 of complement. Such a compact structure is atypical of other multidomain complement proteins so far studied by solution scattering and is fully compatible with mechanisms in which C9 is postulated, on activation, to undergo a drastic unfolding of its domain structure and to expose a more hydrophobic surface which can be embedded into lipid bilayers.

The activation of the complement system in response to the challenge of foreign material in plasma provides a major non-adaptive immune defense mechanism for its host (Reid, 1986; Law & Reid, 1988). Initially, activation proceeds through a series of limited proteolytic steps in one of two largely independent pathways, the classical and alternative pathways. These merge in the lytic pathway, in which the generation of C3b constitutes the essential component of the C5 convertases, which generate C5b from C5. The sequential addition of the late complement components C6, C7, and C8 (a three-chain molecule constructed from C8$\alpha$, C8$\beta$, and C8$\gamma$) and several

* To whom correspondence and requests for reprints should be addressed.
[‡] Royal Free Hospital School of Medicine.
[§] MRC Molecular Immunopathology Unit.

0006-2960/92/0431-754$03.00/0 